Synthesis and Export of the Outer Membrane Lipoprotein in Escherichia coli Mutants Defective in Generalized Protein Export

TAKESHI WATANABE,† SHIGERU HAYASHI, AND HENRY C. WU*
Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

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Export of the outer membrane lipoprotein in Escherichia coli was examined in conditionally lethal mutants that were defective in protein export in general, including secA, secB, secC, and secD. Lipoprotein export was affected in a secA(Ts) mutant of E. coli at the nonpermissive temperature; it was also affected in a secA(Am) mutant of E. coli at the permissive temperature, but not at the nonpermissive temperature. The export of lipoprotein occurred normally in E. coli carrying a null secB::Tn5 mutation; on the other hand, the export of an OmpF::Lpp hybrid protein, consisting of the signal sequence plus 11 amino acid residues of mature OmpF and mature lipoprotein, was affected by the secB mutation. The synthesis of lipoprotein was reduced in the secC mutant at the nonpermissive temperature, as was the case for synthesis of the maltose-binding protein, while the synthesis of OmpA was not affected. Lipoprotein export was found to be slightly affected in secD(Cs) mutants at the nonpermissive temperature. These results taken together indicate that the export of lipoprotein shares the common requirements for functional SecA and SecD proteins with other exported proteins, but does not require a functional SecB protein. SecC protein (ribosomal protein S15) is required for the optimal synthesis of lipoprotein.

Protein export is a complex process which requires the sequential participation of a number of gene products, including the enzymes for the processing of precursor proteins. Genetic analysis has revealed that at least four genes are required for protein export in general: secA, secB, secD, and secY (prlA). secA, secD, and secY mutants have been isolated as conditionally lethal mutants of Escherichia coli and have been found to accumulate precursors of a number of exported proteins at the nonpermissive temperature (4, 14, 18). On the other hand, a null mutation in the secB gene is not lethal, but it affects the export of a subset of outer membrane and periplasmic proteins (9). Mutations in the secC gene have been isolated as suppressor mutations of a secA(Ts) allele (3). secC mutants were found to be cold sensitive in growth; at the nonpermissive temperature, the synthesis of several outer membrane and periplasmic proteins was severely curtailed. It has been proposed that the secC gene product (ribosomal protein S15) interacts with the SecA protein and participates in the coupling of protein synthesis and protein secretion (3).

The major outer membrane lipoprotein differs from most of the other exported proteins in E. coli in that the precursor protein (prolipoprotein) is modified with glycerol and fatty acids, and is subsequently processed by prolipoprotein signal peptidase (SPhase II) during the course of its export. We have previously shown (5) that the export of lipoprotein is affected in secA(Ts) and secY(Ts) mutants, resulting in the accumulation of unmodified prolipoprotein in the cytoplasmic membrane at the nonpermissive temperature. These observations suggest that the early steps in protein export are shared by lipoproteins and nonlipoproteins and that the modification and processing of prolipoproteins represent late events in protein export. On the other hand, we have previously shown (5) that lipoprotein export is not affected in a secB mutant strain.

To further define the export pathway of lipoprotein compared with that of nonlipoproteins in E. coli, we studied lipoprotein export in other mutants that were defective in generalized protein export. In the present study we compared the export of the major outer membrane lipoprotein with those of nonlipoproteins in individual E. coli mutants containing an amber allele of secA, a Tn5 insertion mutation in secB, a cs allele of secC, or a cs allele of secD. Our results indicate that lipoprotein export in E. coli requires functional SecA protein, SecD protein, as well as SecY protein but that it is not affected by the null mutation in secB. In addition, our results indicate that the secC mutation affects the synthesis of lipoprotein and maltose-binding protein (MBP), but does not affect the synthesis of OmpA.

MATERIALS AND METHODS

Phage, bacterial strains, and medium. λD020 phage, which carries the wild-type secA gene, was a generous gift from D. Oliver (State University of New York at Stony Brook, Stony Brook, N.Y.) (15). The bacterial strains used in this study are listed in Table 1. The media used in this study include proteose peptone beef extract (PPBE) broth medium (20) and M9 minimal medium (13) supplemented with thiamine (100 μg/ml), 0.4% glycerol–0.4% maltose, or 0.4% glucose.

Isolation of E. coli (λD020) lysogen. Strain MM52 [secA(Ts)] and strain MM113 [secA(Am)] cells were grown at 30°C overnight in PPBE broth. Five microliters of λD020 phage lysate was spotted onto a lawn of strain MM52 or MM113. Following incubation at 30°C overnight, phage-infected cells from the clear zone were streaked onto a PPBE plate to obtain single colonies. Ten colonies from each strain were tested for immunity against superinfection with a clear
TABLE 1. Bacterial strains and plasmid used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
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<tr>
<td>MC4100</td>
<td>F’ lacU169 relA rpsL thi araD139</td>
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<tr>
<td>MM52</td>
<td>MC4100 secA52(Ts)</td>
<td>J. Beckwith (14)</td>
</tr>
<tr>
<td>MM113</td>
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<td>SFN194</td>
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</tr>
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<td>MM151</td>
<td>MC4100 malT' zhe::Tn10 secB7</td>
<td>J. Beckwith (9)</td>
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<td>MM152</td>
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<td>J. Beckwith (9)</td>
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<td>This study</td>
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<td>MM151W</td>
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<td>This study</td>
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<td>WR19-1</td>
<td>H5C gpaA glpD glpR glpK phoA fade rel-l tonA lpp::Tn10</td>
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<td>MC1000 cs-2124</td>
<td>J. Beckwith (3)</td>
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<td>CG-2</td>
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<tr>
<td>Plasmid pHF100</td>
<td>Amp’ ompF-lpp chimeric gene</td>
<td>S. Mizushima (21)</td>
</tr>
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</table>

Mutant of phage λ, and λDO20 lysogens of strain MM52 and MM113 were obtained.

Construction of strains carrying lpp::Tn10. To eliminate the wild-type lipoprotein structural gene from the secB strain and its parental wild-type strain, strains SFN194W and MM151W carrying lpp::Tn10 were obtained by transduction. The zhe::Tn10 in SFN194 and MM151 were first cured as described by Bochner et al. (1). lpp::Tn10 was then introduced into these cured strains with a Pl1 lysate derived from strain WR19-1, which contained lpp::Tn10. Tet' colonies were analyzed by the Ouchterlony double diffusion test with antiserum against purified lipoprotein and were found to lack lipoprotein. The lpp::Tn10 derivatives of strains SFN194 and MM151 were transformed with plasmid pHF100 (which contained an ompF-lpp hybrid gene and an amp gene), and Tet' Amp' colonies were isolated. These strains were designated SFN194W(pHF100) and MM151W(pHF100), respectively.

Labeling experiments. Pulse-labeling was used to study lipoprotein export in various sec mutants grown at permissive and nonpermissive temperatures. For secA(Ts), secA(Am), and secC mutants, the overnight cultures grown at the permissive temperature were inoculated into fresh M9-glycerol-maltose medium, and the incubation was continued at the permissive temperature. At the mid-logarithmic phase of growth (A<sub>600</sub> 0.4 to 0.5), the cultures were shifted to the nonpermissive temperature; at regular time intervals, 2-ml portions of cultures were withdrawn and added to prewarmed tubes containing 40 μCi of [35S]methionine. After 2 min of labeling with vigorous shaking, trichloroacetic acid (final concentration, 10%) was added and the tubes were immediately placed in an ice bath.

For the secD mutant, labeling was carried out in M9-glycerol-maltose medium for 2 min at both permissive and nonpermissive temperatures with [35S]methionine. For secB mutants, 2 min of labeling with [35S]methionine was used to study lipoprotein export, and 30 s of labeling was used to study the export of the OmpF-Lpp hybrid protein. The trichloroacetic acid precipitates were collected by centrifugation, washed with acetone, and solubilized with 1% sodium dodecyl sulfate (SDS) in 50 mM Tris hydrochloride (pH 8) containing 1 mM EDTA. After this solution was heated at 100°C for 5 min, portions were immunoprecipitated (7) with antisera against lipoprotein, OmpA, MBP, or OmpF. [35S]methionine-labeled immune precipitates were analyzed by SDS-polycrylamide gel electrophoresis (PAGE) for lipoprotein (6) and for other proteins (11). The experimental details regarding incubation temperatures and the length of incubation at the nonpermissive temperature are described in the legends to the figures.

Chemicals. [35S]Methionine (specific activity, 300 Ci/mmol) was purchased from Dupont, NEN Research Products (Boston, Mass). Fixed Staphylococcus aureus cells were purchased from Calbiochem-Behring (La Jolla, Calif.).

RESULTS

Export of lipoprotein in a secA(Am) mutant. It has been shown previously that a secA(Ts) mutant accumulates unmodified prolipoprotein as well as precursors of a number of other exported proteins in the cytoplasmic membrane at the nonpermissive temperature (5, 12). In this study we compared the export of lipoprotein in a secA(Am) mutant (16) with that in the wild-type and a secA(Ts) mutant. When this secA(Am) mutant strain is grown at 42°C, the level of SecA protein is severely reduced, and this reduction in SecA protein results in the failure to synthesize certain exported proteins, such as MBP (16). Significant amounts of pre-MBP and pre-OmpA were detected in the secA(Ts) mutant even at the permissive temperature, but no prolipoprotein was detected (Fig. 1, part 1). This result is in agreement with the previous finding that about 10% of pre-OmpA, but no prolipoprotein, accumulated at 30°C. No accumulation of precursor forms of MBP and OmpA was seen in the wild-type strain. These data suggest that the mutant SecA(Ts) protein is not fully active even at the permissive temperature. Accumulation of prolipoprotein, pre-MBP, and pre-OmpA was observed 2 h after a shift to the nonpermissive temperature, as reported previously (5, 12).

In contrast to the results obtained with the secA(Ts) mutant, there was significant accumulation of prolipoprotein, as well as pre-MBP and pre-OmpA, in the secA(Am) mutant at the permissive temperature (Fig. 1, part 3). Upon a shift to the nonpermissive temperature, lesser accumulation of prolipoprotein and pre-OmpA was observed with increasing time of incubation at this temperature (Fig. 1, part 3). For MBP, an increase in the time of incubation at the nonpermissive temperature resulted in a drastically reduced synthesis of MBP. On the other hand, the defect in the export of MBP, which was manifested by the accumulation of pre-MBP at the permissive temperature, was alleviated after the temperature shift, as was found for lipoprotein and OmpA. These results suggest that there is a general defect in the export of lipoprotein, MBP, and OmpA in the secA(Am)
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FIG. 1. Effect of the wild-type secA gene on the synthesis or export of lipoprotein, OmpA, and MBP in secA(Ts) and secA(Am) mutants of E. coli. MM52 [secA(Ts)], MM52(AO20), MM113 [secA(Am)], and MM113(AO20) cells were grown in M9-glycerol-maltose medium at 30°C and were then shifted to 42°C at the mid-logarithmic phase of growth. Portions (2 ml) were taken from the culture at the time intervals indicated in the figure and were labeled for 2 min with 40 μCi of [35S]methionine. Lipoprotein, MBP, and OmpA were immunoprecipitated with specific antisera, and the immune precipitates were analyzed by SDS-PAGE, as described in the text. (A) MM52 [secA(Ts)]; (B) MM52(AO20); (C) MM113 [secA(Am)]; and (D) MM113(AO20). (A) Lipoprotein; (B) MBP; (C) OmpA protein.

FIG. 2. Lipoprotein export in secB mutants. The wild-type strain SFN194, the secB mutant MM151, and the secB knockout mutant MM152 (secB::Tn5) were grown in M9-glycerol-maltose medium at 30°C. At the mid-logarithmic phase of growth, cells were labeled for 2 min with 40 μCi of [35S]methionine at 30°C. Lipoprotein, MBP, and OmpA protein were analyzed by SDS-PAGE following immunoprecipitation with specific antisera. Abbreviations: PLP, prolipoprotein; LP, lipoprotein; Pre-MBP, pre-maltose-binding protein; MBP, maltose-binding protein; Pre-OmpA, pre-outer membrane protein A; OmpA, outer membrane protein A.

To test this hypothesis, we examined the effect of the wild-type secA gene on lipoprotein export in both secA(Ts) and secA(Am) mutants. The defect in lipoprotein export in the secA(Ts) mutant, which was manifested as the accumu-
ways, as defined by the requirement of the SecB protein, we have studied the effect of the secB mutations on the export of a hybrid protein which comprises the signal sequence of OmpF plus 11 amino acid residues from the mature portion of OmpF and the COOH-terminal 51 amino acid residues from the mature form of the lipoprotein (21). The detailed amino acid sequence around the fusion junction is shown in Fig. 3. This hybrid protein contains an authentic processing site for signal peptidase I (Ala-X-Ala-) of the OmpF protein, but it does not contain the prolipoprotein modification and processing site (Leu-Ala-Gly-Cys-). The molecular weight of the processed form of this hybrid protein is similar to that of lipoprotein, and they migrate to the same position in a SDS-polyacrylamide gel. In order to facilitate the analysis of the hybrid protein in the secB mutant, we constructed derivatives of the wild-type and secB7 mutant strains in which the lpp gene was inactivated by the insertion of Tn10. Plasmid pHF100 carrying the hybrid ompF-lpp gene was then transformed into these strains as described above. The effect of the secB mutation on the export of this hybrid protein was studied by pulse-labeling at 30°C. The export of the OmpF-Lpp hybrid protein was affected by the secB7 mutation, resulting in the accumulation of a significant amount of the precursor of the hybrid protein (Fig. 4B). The export of OmpA and OmpF was not affected by the secB7 mutation (Fig. 4C), while the export of lipoprotein was not affected (Fig. 4A). These results indicate that the SecB protein directly or indirectly interacts with the signal sequence plus 11 amino acid residues of the mature OmpF that is present in the precursor of the Omp-Lpp hybrid protein.

Synthesis of lipoprotein in the secC mutant. It has been reported previously that a mutation in the secC gene affects the synthesis of a number of exported proteins such as MBP, LamB, ribose-binding protein, and OmpF; but it does not affect the synthesis of nonexported proteins such as elongation factor G and SecA at the nonpermissive temperature (23°C) (3). At the nonpermissive temperature (23°C), the relative incorporation of [35S]methionine into periplasmic proteins and outer membrane proteins was reduced to about 40% of that at 37°C in the secC mutant, whereas it was unchanged in the secC+ strain. Moreover, the incorporation of [35S]methionine into soluble proteins and inner membrane proteins was not affected in the secC mutant at the nonpermissive temperature (data not shown).

The effect of the secC mutation on the synthesis of lipoprotein is shown in Fig. 5. While the synthesis of MBP was completely curtailed 1 h after it was shifted to the nonpermissive temperature, the synthesis of OmpA was not affected. Lipoprotein synthesis was significantly affected by

FIG. 3. Structure of OmpF-Lpp hybrid protein. The hybrid protein encoded by the ompF-lpp gene fusion on plasmid pHF100 contained the intact signal sequence of pre-OmpF protein plus 11 amino acid residues of the mature OmpF protein, followed by 51 amino acid residues (+8 to +58) of the mature lipoprotein. Numbers with minus signs indicate the positions of the amino acids in the OmpF signal sequence, and numbers with plus signs indicate those in the mature protein. The processing site in the hybrid protein is presumably the same as that in the wild-type pre-OmpF protein (21).

FIG. 4. Export of OmpF-Lpp hybrid protein in wild-type and secB mutant cells. Strains SFN194 (wild-type), MM151 (secB7 mutant), and SFN194W(pHF100) and MM151W(pHF100) (isogenic strains of SFN194 and MM151, except that they lacked lipoprotein because of lpp::Tn10 and carried the plasmid encoding the OmpF-Lpp hybrid protein) were used to study the effect of the secB mutation on the export of the OmpF-Lpp hybrid protein. Each strain was grown in M9-glycerol-maltose medium at 30°C. At the mid-logarithmic phase of growth, cells were pulse-labeled for 30 s with 40 μCi of [35S]methionine. Lipoprotein and the OmpF-Lpp hybrid protein were immunoprecipitated with antiserum against purified lipoprotein. OmpF and OmpA proteins were immunoprecipitated with the mixed serum of anti-OmpF and anti-OmpA. The proteins were analyzed by SDS-PAGE after immunoprecipitation. (A) Lipoprotein (LP), SFN194 (secB+), and MM151 (secB); (B) OmpF-Lpp hybrid protein, SFN194W(pHF100) (secB+), and MM151W(pHF100) (secB); (C) OmpF and OmpA proteins, SFN194W(pHF100) (secB+), and MM151W(pHF100) (secB). PLP, Prolipoprotein
the secC mutation, albeit not as severely as that of MBP (Fig. 5B). In the wild-type strain, there was no change in the synthesis of MBP, OmpA, and lipoprotein on a shift to 23°C (Fig. 5A). These results indicate the selective nature of the secC mutation in affecting the synthesis of exported proteins; it affects the synthesis of the constitutively expressed lipoprotein as well as the inducible MBP, albeit to different extents, but has little effect on the synthesis of OmpA.

Export of lipoprotein in the secD mutation. The secD mutant is cold sensitive for growth and accumulates precursor forms of several exported proteins such as alkaline phosphatase, MBP, OmpF, and ribose-binding protein at the nonpermissive temperature (23°C) (4). We studied the effect of the secD mutation on the lipoprotein export. The export of OmpA and MBP was affected in the secD mutant at the nonpermissive temperatures (Fig. 6), resulting in the accumulation of the precursor forms. The export of lipoprotein was also affected by the secD mutation, albeit to a lesser extent.

DISCUSSION

In the present study we investigated the export of lipoprotein in various E. coli mutants that were defective in protein export in general. Lipoprotein export differs from the export of other precursor proteins in that it is processed by a distinct signal peptidase, prolipoprotein signal peptidase, or SPase II. The processing of prolipoprotein is preceded by a series of modification reactions which convert the unmodified prolipoprotein to glyceride-modified prolipoprotein. Inasmuch as modification and processing of prolipoprotein represent late events in the export of lipoprotein to the outer membrane, it is reasonable to assume that the early steps in the export process are shared by precursors of lipoproteins and nonlipoproteins. Based on results of this study, together with earlier findings that the export of lipoprotein is affected in secA and secY mutants, we conclude that three of the four genes required for protein export in general are also essential for the export of lipoprotein in E. coli, i.e., secA, secD, and secY.

The requirement of a functional SecB gene product divides the exported proteins into two groups: secB-dependent and secB-independent proteins. Lipoprotein appears to be long to the latter group. A functional SecB protein is required, however, for the optimal export of a hybrid protein consisting of the signal sequence plus 11 amino acid residues of OmpF and the mature protein of lipoprotein. This hybrid protein is presumably processed by SPase I due to the absence of the modification and processing site unique to prolipoprotein (21). The requirement of the SecB protein for the export of this hybrid protein cannot be attributed to a change in the specificity of the processing enzyme from SPase II to SPase I, since precursors of other such secB-independent proteins as ribose-binding protein and alkaline phosphatase are also processed by SPase I. Our results suggest that the SecB protein interacts directly or indirectly with the precursor of the OmpF-Lpp hybrid protein, presumably via the signal sequence and the 11 amino acid residues of OmpF. Since the defect in the export of the hybrid protein is quantitatively much less than that of OmpF, the putative interaction of the SecB protein with the precursors of the exported proteins must be dependent on the overall structure and conformation of the precursor protein rather than on that of the signal sequence of the precursor protein alone.

We also showed that the synthesis of lipoprotein, which is constitutive rather than inducible, is also affected by the mutation in the secC gene, as was found for the synthesis of an inducible exported protein, MBP. On the other hand, synthesis of OmpA was not affected by the secC mutation. Thus, the apparent requirement of a functional SecC protein further divides the exported proteins into two subsets: secC-dependent and secC-independent proteins. The role of the SecC protein (ribosomal protein S15) in the synthesis of exported proteins or in the coupling of protein synthesis with protein export remains unclear.

The notion that the synthesis of an exported protein is somehow coupled to its export is not only implicit in the revised model of signal hypothesis vis à vis the signal recognition particle in the eucaryotic cell but it is also suggested by results of earlier studies on the synthesis of MBP in secA(Am) mutants of E. coli (16). Results of our studies on the synthesis and export of lipoprotein, OmpA, and MBP in secA(Ts) and secA(Am) mutants revealed a rather complicated picture. Our results suggest that a functional SecA protein is essential for the export of all three
proteins. This conclusion is based on the finding that precursors of MBP, OmpA, and lipoprotein accumulated in the secA(Ts) mutant at the nonpermissive temperature and in the secA(Am) mutant at the permissive temperature. Similar observations have been reported by Liss and Oliver (12). The unexpected finding in this study was the decrease in the accumulation of all three precursor proteins in the secA(Am) mutant at the nonpermissive temperature. This observation is consistent with the notion that the export of lipoprotein and OmpA requires lesser amounts of the functional SecA protein than other exported proteins. It has been noted previously that the secA(Ts) mutation does not affect the synthesis of MBP compared with the secA(Am) allele (12, 14, 16). It has been suggested that these two secA mutations may only differ quantitatively, with the secA(Am) mutation being a tighter one than the secA(Ts) mutation (19). Our results on the defective export of lipoprotein and OmpA in secA(Ts) and secA(Am) mutants do not support this interpretation. The different phenotypes of the secA(Ts) and secA(Am) mutants remain to be explained.

While the defect in the export of lipoprotein, MBP, and OmpA in the secA(Am) mutant was complemented by the wild-type secA gene, the defect in the synthesis of MBP in the secA(Am) mutant at the nonpermissive temperature was not restored in the lysogen containing λDO20. Results of previous studies (19) have led to the conclusion that the inhibition of MBP synthesis in the secA(Am) mutant at the nonpermissive temperature is a secondary consequence of the primary export defect, since the addition of cyclic AMP reverses the synthesis defect without affecting the export defect of MBP in the secA(Am) mutant. Our data show that in the presence of the wild-type secA allele, the synthesis block remains while the export defect is restored. Thus, the reduction in the synthesis of MBP in the putative secA(Am) mutant is not a consequence of the export defect in this mutant; rather, it may be a consequence of a defect in a neighboring gene adjacent to the secA gene, the expression of which is reduced by the amber mutation because of a polarity effect. Further work is needed to clarify this paradox.

Figure 7 summarizes our current understanding of the export of lipoproteins, as defined by the requirements of the gene products encoded by secA, secB, secC, and secD compared with the requirements of such nonlipoproteins as OmpA, OmpF, and MBP. It is clear that further work in which both genetic and biochemical approaches are used will be needed to elucidate further the export pathway of proteins in E. coli.

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LITERATURE CITED

17. Rotering, H., W. Fiedler, W. Rolllinger, and V. Braun. 1984. Procedure for the identification of Escherichia coli mutants affected in components containing glycerol derived from phos-


